The Journal of Experimental Biology 203, 1123–1130 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
JEB2627

DEPRESSION OF NUCLEAR TRANSCRIPTION AND EXTENSION OF mRNA HALF-LIFE UNDER ANOXIA IN *ARTEMIA FRANCISCANA* EMBRYOS

FRANK VAN BREUKELEN*, RYAN MAIER AND STEVEN C. HAND‡

Section of Integrative Physiology and Neurobiology, Department of Environmental, Population, and Organismic Biology, University of Colorado, Boulder, CO 80309-0334, USA

*Present address: Department of Cellular and Structural Biology, University of Colorado School of Medicine, Denver, CO 80262, USA
‡Author for correspondence (e-mail: Hands@spot.colorado.edu)

Accepted 1 February; published on WWW 9 March 2000

Summary

Transcriptional activity, as assessed by nuclear run-on assays, was constant during 10 h of normoxic development for embryos of the brine shrimp *Artemia franciscana*. Exposure of embryos to only 4 h of anoxia resulted in a 79.3±1 % decrease in levels of *in-vivo*-initiated transcripts, and transcription was depressed by 88.2±0.7 % compared with normoxic controls after 24 h of anoxia (means ± S.E.M., *N*=3). Initiation of transcription was fully restored after 1 h of normoxic recovery. Artificially lowering the intracellular pH of aerobic embryos to the value reflective of anoxia (pH 6.7) showed that acidification alone explained over half the transcriptional arrest. Initiation of transcription was not rescued by application of 80 % carbon monoxide under anoxia, which suggests that heme-based oxygen sensing is not involved in this global arrest. When these transcriptional data are combined with the finding that mRNA levels are unchanged for at least 6 h of anoxia, it is clear that the half-life of mRNA is extended at least 8.5-fold compared with that in aerobic embryos. In contrast to the activation of compensatory mechanisms to cope with anoxia that occurs in mammalian cells, *A. franciscana* embryos enter a metabolically depressed state in which gene expression and mRNA turnover are cellular costs apparently not compatible with survival and in which extended tolerance supercedes the requirement for continued metabolic function.

Key words: gene expression, mRNA, transcription, anoxia, oxygen sensing, brine shrimp, *Artemia franciscana.*

Introduction

While physiological responses to oxygen deprivation among animals are diverse (Hochachka et al., 1993, 1996; Grieshaber et al., 1994; Somero, 1998), the resulting tolerance to this stress depends critically on the degree of metabolic depression achievable (Hochachka and Guppy, 1987; Hand, 1998). The greater the downregulation of ATP turnover, the longer the anoxic bout that can be tolerated. Short-term responses to hypoxia in mammalian cells can involve upregulation of selected suites of genes, such as those encoding for the enzymes of glycolysis (Semenza et al., 1994; Dang and Semenza, 1999). However, animals with the greatest anoxia tolerance are found among invertebrate phyla (Grieshaber et al., 1996; Hand and Hardewig, 1996), and many of these species downregulate metabolism to low levels. Considering the implication for energy-consuming processes, one might predict that nuclear transcription would be arrested and this, if true, would represent a markedly different strategy to cope with anoxia compared with that of vertebrates. In this study, we have employed nuclear run-on assays to evaluate transcriptional activity under anoxia in embryos of the brine shrimp *Artemia franciscana*, which is one of the most anoxia-tolerant animals known.

In response to anoxia, the encysted gastrula-stage embryos of *A. franciscana* enter a reversible state of developmental and metabolic quiescence that can last for 4 years or more (Clegg, 1997; for reviews of quiescence, see Hochachka and Guppy, 1987; Storey and Storey, 1990; Hand, 1991, 1998; Guppy and Withers, 1999; Hand and Hardewig, 1996; Hand and Podrabsky, 1999). Many catabolic and anabolic processes are depressed, resulting in energy flows that are very low compared with aerobic values (Hand and Gnaiger, 1988; Hontoria et al., 1993; Hand, 1995). The embryos may even approach an ametabolic state (Clegg, 1997). Considerable experimental evidence shows that the rapid and acute acidification of intracellular pH (from pH 7.7–7.9 to pH$\leqslant$6.7) that occurs as these embryos enter anoxia (Busa at al, 1982; Kwast et al., 1995; Clegg et al., 1995) plays a key role in the metabolic arrest. A depression of protein synthesis occurs during anoxia in *A. franciscana* embryos (Clegg and Jackson, 1989; Hofmann and Hand, 1990, 1994) that is mediated through translational control. However, the fate of transcription during anoxia-induced quiescence is unknown.

Hardewig et al. (1996) demonstrated that the ontogenetic increase in levels of actin mRNA is prevented by exposure to anoxia and that the levels of this message are stable for several

hours of anoxia, as is the case for total polyadenylated mRNA (Hofmann and Hand, 1992). One explanation for these patterns could be simultaneous arrest of nucleic acid synthesis and degradation, similar to that documented for protein degradation in these embryos under anoxia (see Anchordoguy et al., 1993; Anchordoguy and Hand, 1994, 1995). Transcription accounts for a modest proportion of the cellular energy budget (typically 1–10 % of basal metabolic rate in mammalian cells; Rolfe and Brown, 1997), but considering the large metabolic depression required to survive prolonged anoxia, even a 1 % drain on cellular energy stores might not be sustainable.

RNA synthesis can be readily detected in *A. franciscana* embryos during active development under normoxic, fully hydrated conditions (Clegg and Golub, 1969). These gastrula-stage embryos possess predominantly RNA polymerase II activity and some RNA polymerase I activity, but apparently little RNA polymerase III activity (D'Alessio and Bagshaw, 1977; Hentschel and Tata, 1977). In the present study, transcriptional activity was measured in nuclei isolated from both normoxic and anoxic embryos and from those exposed to aerobic acidosis and to carbon monoxide under anoxia. Nuclear run-on assays offer the advantage of providing a snapshot of the levels of *in-vivo*-initiated transcripts at the moment of nuclear isolation (e.g. Stallcup et al., 1978). Administration of carbon monoxide during hypoxia is known to blunt oxygen-dependent gene expression, an action mediated by the binding of CO to heme groups, which thereby locks the putative hemoprotein sensor (Goldberg et al., 1988; Acker, 1994; Ratcliffe et al., 1998) in the oxy conformation. Our evidence demonstrates that an acute, global arrest of transcriptional initiation occurs during anoxia in *A. franciscana* embryos, and that this is not reversed by supplying carbon monoxide under oxygen-free conditions.

Materials and methods

Preparation and incubation of embryos

Brine shrimp (*Artemia franciscana*) embryos were purchased from Sander's Brine Shrimp Co. (Ogden, UT, USA). The hatching percentage for these embryos is approximately 80 % (Anchordoguy et al., 1993). Embryos were hydrated for at least 4 h at 0 ° C, washed in a solution of 0.25 mol l^{-1} NaCl, and then dechorionated as described previously (Kwast and Hand, 1993). The embryos were allowed to develop for 4 h under normoxia (0.25 mol l^{-1} NaCl solution; air-saturated, 22–23 °C) and then incubated in media equilibrated with one of the desired gas mixtures (anoxia, 100 % Ar or N_2; aerobic acidosis, 60 % CO_2:40 % O_2; carbon monoxide, 80 % CO in oxygen-free nitrogen). The carbon monoxide was obtained pre-mixed from Matheson Gas Products (Cucamonga, CA, USA). Recovery from anoxia was achieved simply by returning embryos to normoxic incubation conditions. At the end of each treatment regime, embryos were chilled to 0 ° C while still immersed in the appropriate gas-equilibrated medium. The embryos were then filtered through Dura Wipe cloth (Johnson and Johnson Advanced Materials Company, New Brunswick, NJ, USA), rapidly transferred to ice-cold homogenization medium (HM) and processed.

Nuclear isolation

RNAase-free conditions were maintained throughout the nuclear isolation and run-on assays. Nuclei were isolated using the method of Acey et al. (1994) with modification. Hydrated and dechorionated embryos (5 g) were homogenized with two passes of a Teflon/glass homogenizer (model 3431; Thomas Scientific, Swedesboro, NJ, USA) in 8 volumes of ice-cold HM consisting of 10 mmol l^{-1} Tris-HCl buffer, pH 7.5, 10 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} NaCl, and 0.1 % Nonidet P-40 (NP-40; Igepal CA-630, Sigma Chemical Co., St Louis, MO, USA). The pestle was motor-driven at approximately 500 revs min^{-1}. The homogenate was filtered through one layer of Dura Wipe cloth and centrifuged at 800 ***g*** for 10 min at 2 °C. The pellet was gently resuspended in 8 ml of HM without NP-40, and 2 ml samples were layered onto each of four Percoll pads (12 ml of 75 % Percoll containing 150 mmol l^{-1} NaCl, 10 mmol l^{-1} $MgCl_2$ and 10 mmol l^{-1} Tris-HCl, pH 7.5) contained in 50 ml centrifuge tubes. The samples were centrifuged in a fixed-angle rotor at 15 000 ***g*** for 15 min at 2 °C. Nuclei accumulated at the sample/Percoll interface, while yolk platelets sedimented through the Percoll to the bottom of the tubes. Nuclei were collected by aspiration, diluted in approximately 5 volumes of HM without NP-40 and recentrifuged at 850 ***g*** for 10 min at 2 °C. The washing procedure was then repeated once to ensure removal of all the Percoll. The final nuclear pellets were resuspended in approximately 1 volume of glycerol storage buffer (50 mmol l^{-1} Tris-HCl, pH 8.3, 40 % glycerol, 5 mmol l^{-1} $MgCl_2$ and 0.1 mmol l^{-1} EDTA) and frozen at –80 °C until use. To estimate nuclear concentration, nuclei were stained with 0.01 % Methyl Green and quantified with an improved Neubauer hemocytometer (C. A. Hausser & Son, Philadelphia, PA, USA). The typical yield from 5 g of dechorionated embryos was approximately 400×10^6 to 500×10^6 nuclei.

Transcriptional run-on assays

Unless indicated otherwise, run-on assays (modified from Acey et al., 1994) were performed under conditions optimized for nuclear concentration, pH and nucleotide concentrations. The optimized reaction mixture (total assay volume 209.5 µl) contained: 100 µl of a buffer solution [100 mmol l^{-1} Tris, 100 mmol l^{-1} maleate, 150 mmol l^{-1} KCl, 40 mmol l^{-1} $(NH_4)_2SO_4$, 4.8 mmol l^{-1} magnesium acetate and 4 mmol l^{-1} $MnCl_2$, pH 7.6], 15 µl of a nucleotide mixture [3.3 mmol l^{-1} ATP, 6.7 mmol l^{-1} CTP, 6.7 mmol l^{-1} GTP], 0.5 µl of 20 mmol l^{-1} UTP, 5 µl of 111 TBq $mmol^{-1}$ [^{32}P]UTP (185 MBq; DuPont NEN Life Science Products, Boston, MA, USA), 3 µl of 1 mol l^{-1} dithiothreitol in 0.01 mol l^{-1} sodium acetate, pH 5.2, 2 µl of placental RNasin (10 units total; Promega, Madison, WI, USA), 4 µl of water and 80 µl of a nuclear suspension containing 650×10^6 to 800×10^6 nuclei ml^{-1}. Assays were initiated by the addition of nuclei, and the reaction mixtures were incubated at 30 °C for the indicated times. Triplicate samples (30 µl each) were removed at the selected

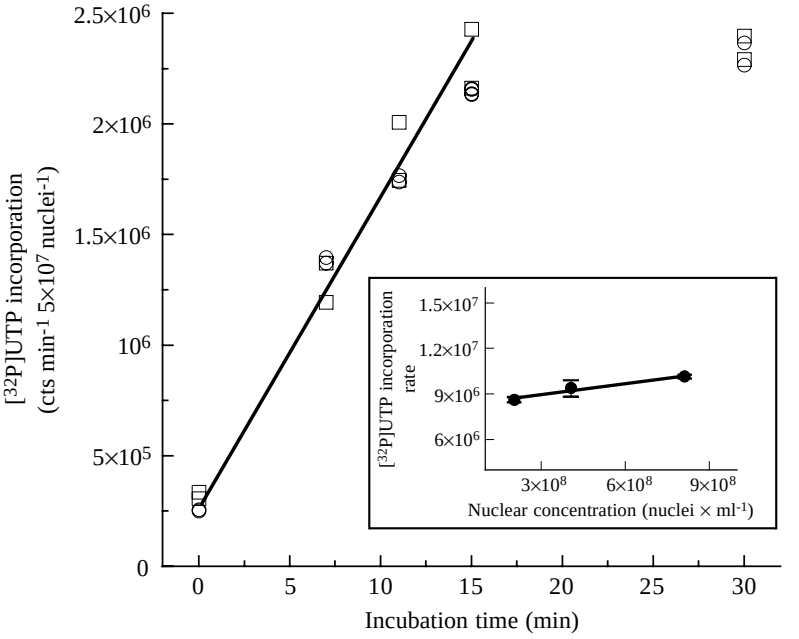

Fig. 1. Time course of incorporation of [^{32}P]UTP by isolated nuclei from *Artemia franciscana* embryos. Subsequent assays were restricted to 15 min. Inset: the effect of nuclear concentration on transcriptional rate expressed per hour (the slope does not differ significantly from zero, $P>0.05$). Values represent means ± S.E.M., $N=3$.

time points and applied to Whatman GF/C glass fiber filters (Whatman International Ltd, Maidstone, UK; 2.4 cm). The filters were immediately transferred to ice-cold 10 % trichloroacetic acid (TCA) containing 1 % sodium pyrophosphate and soaked for 1 h with stirring. The filters were then given two successive washes in 5 % TCA with 0.5 % pyrophosphate for 10 min each, followed by three 10 min washes in 95 % ethanol. During all washing procedures, the filters were kept in perforated plastic containers to prevent mechanical damage. The filters were dried in air. Radioactivity was determined by liquid scintillation counting in Scintiverse II fluor (Fisher Scientific, Denver, CO, USA) and expressed as disints min^{-1}. Values at time zero were subtracted from values at later time points to correct for non-specific binding of radioactivity to the filters. Data were analyzed by analysis of variance (ANOVA) or regression analysis where appropriate. Values are presented as means ± S.E.M.

For the determination of apparent Michaelis–Menten constants (K_m), the concentration of one nucleotide was varied while the other nucleotide concentrations were held constant at 240 μmol l^{-1} ATP, 480 μmol l^{-1} CTP, 480 μmol l^{-1} GTP and 48 μmol l^{-1} UTP. When UTP concentration was varied, the specific radioactivity of UTP was maintained at 185 MBq μmol^{-1}. K_m values were estimated by nonlinear regression analysis using the Leonora program (Cornish-Bowden, 1995). The pH profile for elongation was generated by adjusting the pH of the Tris-maleate reaction buffer and then verifying the pH values of the final reaction mixtures by direct measurement in parallel samples.

Results

Transcription by isolated nuclei and optimization of run-on conditions

Nuclear run-on assays quantify the elongation of *in-vivo*-initiated transcripts. After cell lysis, initiation of new transcripts is negligible (see Weber et al., 1977; Groudine et al., 1981; Hofer and Darnell, 1981; Lohr and Ide, 1983). Thus, the level of transcriptional initiation present at the time of cell lysis can be compared among organismic treatment groups. As is shown in Fig. 1, incorporation of [^{32}P]UTP by isolated nuclei from *A. franciscana* embryos was linear for 15 min. Consequently, subsequent assays were restricted to this period. UTP incorporation in these assays was inhibited by 81 % by adding 50 μg ml^{-1} actinomycin D (data not shown). When isolated nuclei were serially diluted across a fourfold range of nuclear concentration (Fig. 1, inset), there was no significant difference in transcriptional activity when expressed as a function of nuclear density (the slope is not significantly different from zero, $P>0.05$).

Elongation of the transcripts was dependent on ATP, CTP, GTP and UTP concentration. For all four precursors of RNA synthesis, transcription followed Michaelis–Menten saturation kinetics (Fig. 2A–D). Apparent K_m values (μmol l^{-1} ± S.E.M. of kinetic parameter) were 5.0±0.8, 2.9±0.1, 5.9±0.5 and 6.9±1.3 μmol l^{-1} for ATP, CTP, GTP and UTP, respectively. Our apparent K_m values for nuclei from *A. franciscana* embryos closely match other published values for isolated nuclei, which fall within the range 1–10 μmol l^{-1} (Furuichi, 1981; Job et al., 1988; Klumpp et al., 1998).

Nuclei isolated from normoxic embryos were used to determine the pH sensitivity for transcriptional elongation (Fig. 3). When pH was reduced from 7.9, the value approximating the intracellular pH (pHi) of aerobic embryos, to pH 6.7–6.8 (reflective of the value during early quiescence; Busa et al., 1982; Kwast et al., 1995), elongation was inhibited by 50 %. If assay pH was lowered further to 6.3, elongation rate was inhibited by 65 %. The latter pH value is physiologically relevant because it is observed *in vivo* after an overnight exposure of embryos to anoxia (Busa et al., 1982).

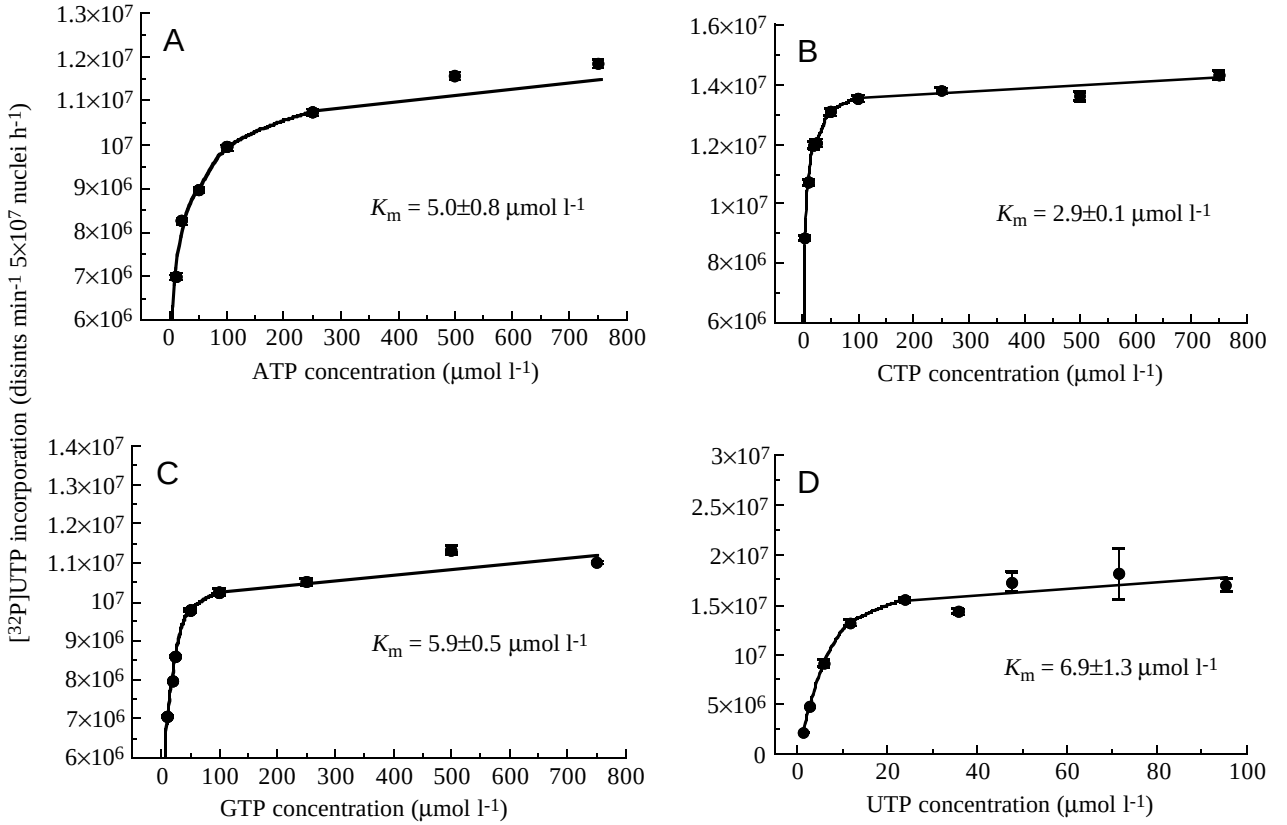

Fig. 2. Substrate saturation profiles for ATP (A), CTP (B), GTP (C) and UTP (D) during transcriptional elongation. Values represent means ± S.E.M., N=3.

Arrest of transcriptional initiation in vivo

Transcriptional activity in *A. franciscana* embryos during pre-emergence development was unchanged over 10 h at room temperature (22–23 °C) under normoxia (Fig. 4). The slope of the relationship between UTP incorporation rate by isolated nuclei *versus* time of embryonic development was not statistically different from zero (P>0.05). However, when aerobic development was interrupted by exposing embryos to 4 h of anoxia (Fig. 5), transcriptional activity was reduced by 79.3±1 % (mean ± S.E.M., N=3; analysis of covariance, P<0.05). When the duration of the anoxic bout was extended to 24 h, transcriptional activity was depressed a maximum of 88.2±0.7 % (mean ± S.E.M., N=3). Transcriptional activity was fully restored after anoxia by allowing the embryos 1 h of normoxic recovery (Fig. 5).

Incubation of embryos under the artificial condition of aerobic acidosis (60 % CO_2:40 % O_2) provides insight into the role of pHi acidification in the arrest of transcription. This treatment is known to depress the pHi of embryos to 6.8 (Busa and Crowe, 1983) under fully aerobic conditions, a value comparable with that measured in embryos after 1 h of anoxia. Intracellular ATP concentration remains high and unchanged for several hours of aerobic acidosis, segregating this factor from pHi (Carpenter and Hand, 1986; Anchordoguy and Hand, 1995). When nuclei were isolated from embryos given 4 h of normoxic incubation followed by an additional 4 h exposure to aerobic acidosis, the initiation of transcription was depressed by 55.2±3.6 % compared with the normoxic value (Fig. 6A; ANOVA, P<0.05). Hyperoxia experienced by embryos during the aerobic acidosis treatment had no significant effect on transcription; UTP incorporation was identical for embryos incubated under normoxia for 4 h (air-saturated medium) and in those incubated in medium equilibrated with 60 % N_2:40 % O_2 (ANOVA,

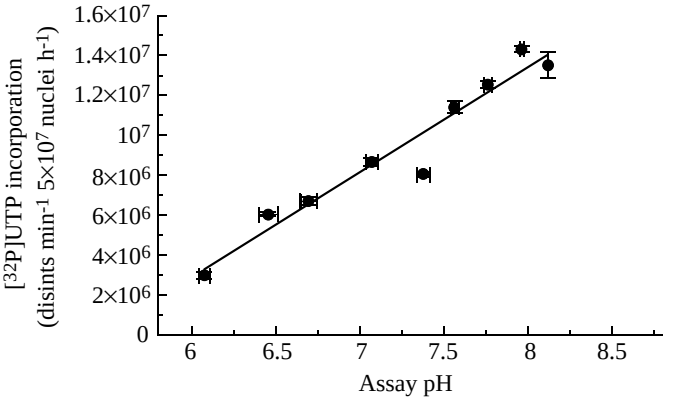

Fig. 3. pH profile for transcriptional elongation in isolated nuclei from *Artemia franciscana* embryos. pH values (horizontal error bars) for each mean reflect those measured in reaction mixtures. Values are means ± S.E.M., N=3. The line was fitted by linear regression (r^2=0.94, P≤0.05).

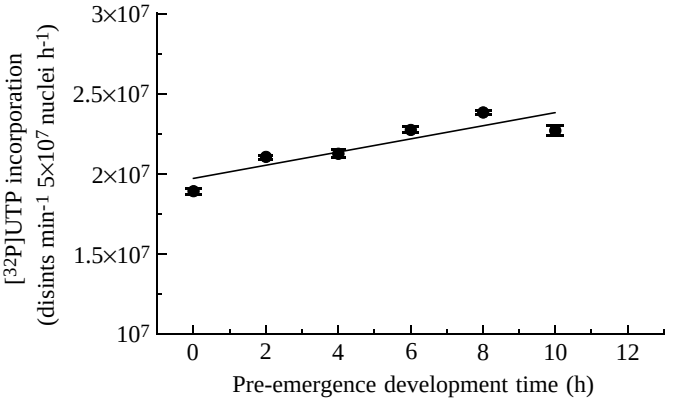

Fig. 4. Transcription rate in nuclei isolated from *Artemia franciscana* embryos as a function of time during pre-emergence development (i.e. prior to the emergence of nauplius larvae). Values are means ± S.E.M., N=3. The line was fitted by linear regression, and the slope was not significantly different from zero (P>0.05).

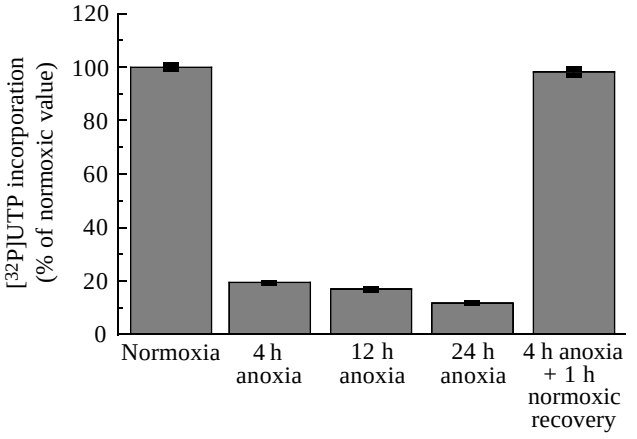

Fig. 5. Arrest of transcriptional initiation due to exposure of *Artemia franciscana* embryos to anoxia for the indicated times. Embryos received a 4 h incubation under normoxia prior to anoxic bouts, and complete recovery was accomplished by returning embryos to normoxia. Transcriptional activity was measured in isolated nuclei. Values represent means ± S.E.M., N=3.

P>0.05; data not shown). Thus, over half of the transcriptional arrest under anoxia can be ascribed to acidification of pHi.

To evaluate the involvement of a heme-based oxygen sensor in the global depression of transcription seen under anoxic exposure, embryos were incubated in the presence of 80 % CO:20 % N_2. Ligation of the porphyrin heme with CO is sufficient to convert hemoproteins to the oxy conformation in the absence of oxygen (Goldberg et al., 1988). Thus, if a heme-based oxygen sensor were involved in the global depression of transcription under anoxia, the addition of 80 % carbon monoxide to anoxic embryos would be expected to reverse at least some of the transcriptional depression. However, the addition of carbon monoxide to anoxic embryos was without effect on transcription, i.e. it did not reverse the depression caused by anoxia alone (Fig. 6B; ANOVA, P>0.05).

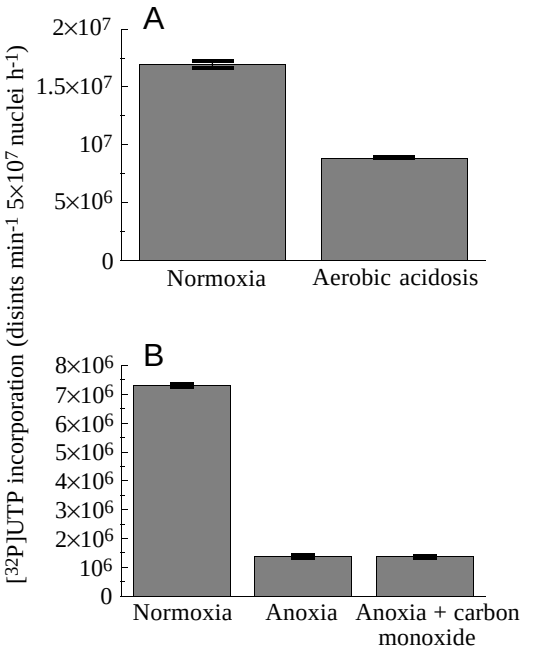

Fig. 6. (A) Depression of transcription induced by incubating *Artemia franciscana* embryos under the artificial condition of aerobic acidosis (60 % CO_2:40 % O_2) for 4 h compared with transcription by embryos given 4 h of normoxia alone. Transcriptional activity was measured in isolated nuclei. (B) Exposure of embryos to anoxia (medium equilibrated with 100 % N_2) compared with transcriptional activity of embryos given carbon monoxide under anoxia (medium equilibrated with 80 % CO:20 % N_2). All values represent means ± S.E.M., N=3.

Discussion

In contrast to recent work with mammalian systems in which upregulation of selected genes during oxygen-limitation is prevalent (see Bunn and Poyton, 1996; Semenza et al., 1994; Dang and Semenza, 1999), exposure of *A. franciscana* to anoxia elicits a global arrest of nuclear transcription. Instead of activating compensatory mechanisms to cope with anoxia, this animal enters a state of deep metabolic depression during which gene expression represents a cellular cost that is apparently not compatible with survival. When embryos were exposed to anoxia, transcriptional initiation was reduced by approximately 90 % relative to normoxic controls. This *in vivo* downregulation of initiation occurs rapidly and, to our knowledge, is the largest transcriptional arrest reported to date in response to oxygen limitation. The elongation of these transcripts was performed in our experiments under optimal assay conditions (i.e. high pH and nucleotide availability) but, as discussed below, the actual transcriptional activity under anoxia may be even lower than estimated if pausing of the polymerase during elongation is also promoted because of the low pH and ATP availability characteristic of this state in these embryos.

Run-on assays performed with nuclei isolated from aerobic acidotic embryos revealed a 55 % reduction in the initiation of transcription relative to normoxic controls (Fig. 6A), thereby

indicating a clear role for acidification in the inhibition of initiation. In addition to a direct influence on the transcriptional machinery, the proximal effect of pH acidification on initiation could involve the recently reported pH-dependent translocation of p26, a small heat-shock protein, to the nuclear compartment of *A. franciscana* embryos (Clegg et al., 1994; Liang et al., 1997a; Liang and MacRae, 1999). Under anoxia, approximately half the total embryonic p26 is transported from the cytoplasm to the nucleus, and this process is reversed during aerobic recovery. Acidic pH favors the accumulation of p26 in the nucleus, and realkalization of embryos favors the removal of p26 from the nucleus. While the protein clearly possesses molecular chaperone activity (Liang et al., 1997b), it may well be that this protein is involved in some manner in the transcriptional arrest reported in the present study (see Clegg et al., 1995). It is appropriate to note that the p26 is accumulated in the encysted embryo to levels that represent 15 % of the total non-yolk protein (Clegg et al., 1994). Regardless of the mechanism by which pHi affects transcription, the results obtained under aerobic acidosis indicate that other factors are required to explain fully the depression of transcriptional initiation seen *in vivo* during anoxia.

Another hallmark change in *A. franciscana* embryos known to occur sufficiently rapidly to be of relevance for the transcriptional arrest observed in this study is the level of cellular ATP. While ATP concentration is well-regulated in most mammalian systems and major declines are usually only associated with cell death (Atkinson, 1977), anoxic *A. franciscana* embryos experience a large reduction in ATP concentration (over 80 %) during the first hour of anoxia (Stocco et al., 1972; Carpenter and Hand, 1986; Rees et al., 1989; Anchordoguy and Hand, 1994). Indeed, part of the acidification in pHi observed during early quiescence is caused by net ATP hydrolysis (see Kwast et al., 1995 and references therein). Data for bacterial transcription suggest that initiation at the rRNA promoter is dependent on high nucleoside triphosphate concentrations, such that growth-rate-dependent changes in ATP and GTP concentrations may eventually determine rates of ribosome synthesis (Gaal et al., 1997). Furthermore, RNA synthesis in Ehrlich ascites cells is also apparently influenced by ATP and GTP levels (Grummt and Grummt, 1976). On the basis of these considerations, it is likely that the low ATP levels in the absence of oxygen explain a sizable proportion of the *in vivo* arrest of transcriptional initiation in *A. franciscana* embryos.

While many, perhaps most, changes in transcriptional rate are due to depressed initiation (see Egan et al., 1994; Lassam and Jay, 1989; Spencer and Kilvert, 1993), recent work has shown that pausing of the polymerase during elongation may limit the expression of many genes (e.g. *β-actin*, *c-fos*, *c-myc*, *Hsp70*; for a review, see Uptain et al., 1997). Elongational arrest within coding regions can effectively depress mRNA synthesis from the affected genes (Shilatiford, 1998) and, in principle, arrest of elongation and initiation are not mutually exclusive. In *A. franciscana* embryos, the large changes in pHi and ATP availability under anoxia may work together to downregulate both processes. If re-entry of paused polymerases into productive elongation occurs when isolated nuclei are placed in optimized conditions for run-on assays, then elongational pause would go undetected and the *in vivo* arrest of transcription would be even greater than suggested by the results presented in Fig. 5.

In attempts to identify other factors involved in the arrest of transcription in quiescent embryos, we also considered the activity of heme-based oxygen sensors. Kwast and Hand (1996a,b) provided evidence for a mitochondrially located oxygen sensor that may mediate translational arrest within this organelle during anoxia in *A. franciscana* embryos. A sizable literature now suggests that the nuclear transcription of many hypoxia-inducible genes is regulated *via* heme-dependent oxygen sensing (Bunn and Poyton, 1996; Dang and Semenza, 1999; Land and Hochachka, 1995). One test for the involvement of an oxygen sensor is the ability to reverse the effects of hypoxia/anoxia on transcription by administering carbon monoxide (Goldberg et al., 1988). In the case of *A. franciscana* embryos under anoxia, CO-induced blunting of the anoxic effect on transcription would mean a deinhibition of the process. However, the addition of 80 % carbon monoxide to anoxic embryos was without effect and did not rescue transcriptional initiation (Fig. 6B). Thus, it seems unlikely that a heme-based oxygen sensor is pivotal in the global inhibition of nuclear transcription observed during quiescence. One might argue, however, that the CO treatment was ineffective under anoxia because pHi and adenylate levels were simply too low *in vivo* to permit activation of transcription even if a putative oxygen sensor had been shifted to the oxy conformation by CO binding. Perhaps it is under hypoxia rather than anoxia where oxygen sensing may play an observable role in *A. franciscana* embryos.

An additional conclusion that emerges from this study, when combined with our results that show unchanged mRNA concentrations for at least the first 6 h of anoxia (Hofmann and Hand, 1992; Hardewig et al., 1996), is that mRNA half-life ($t_{1/2}$) is extended in the anoxic embryo by at least 8.5-fold compared with the normoxic embryo [where $t_{1/2}$=[RNA]×$\log_e$2×(synthesis rate)$^{-1}$]. mRNA pools in the embryo would decline markedly if message stability were not increased simultaneously in the face of the transcriptional arrest during anoxia. One biological advantage of stable mRNA pools under anoxia would be the quick resumption of protein synthesis as soon as oxygen is returned to the embryo.

In summary, the depression of transcription and the extension of mRNA half-life are concordant with limited energy availability. Transcriptional downregulation is facilitated by the decreases in pHi and ATP concentration that occur under anoxia and their resulting influence on initiation and perhaps elongation. At this juncture, the available evidence does not indicate the involvement of a hemoprotein oxygen sensor that downregulates nuclear transcription. In vertebrates, the upregulation of critical suites of genes serves a homeostatic role during anoxic bouts of limited duration. In contrast, the

global arrest seen in *A. franciscana* precludes normal metabolic functioning, but it extends the tolerance to anoxia dramatically, which may be more critical when the variable physical environment of these animals is considered. Multiple cellular solutions for coping with oxygen limitation, each with inherent biological tradeoffs, are available across animal phyla.

This work was supported by National Science Foundation grant IBN-9723746 to S.H. and by an EPO Biology Fellowship and Graduate School Dean's Small Grant Award to F.v.B. We would like to thank Dr Mike Grant for assistance with statistical analyses and Dr Roger Acey for advice on protocols for nuclear isolation and run-on assays.

References

Acey, R. A., Tran, K. Q. and Bagshaw, J. C. (1994). Isolation of transcriptionally active nuclei from *Artemia*. *Mol. Biol. Cell* **5**, 226A.

Acker, H. (1994). Mechanisms and meaning of cellular oxygen sensing in the organism. *Respir. Physiol.* **95**, 1–10.

Anchordoguy, T. J. and Hand, S. C. (1994). Acute blockage of the ubiquitin-mediated proteolytic pathway during invertebrate quiescence. *Am. J. Physiol.* **267**, R895–R900.

Anchordoguy, T. J. and Hand, S. C. (1995). Reactivation of ubiquitination in *Artemia franciscana* embryos during recovery from anoxia-induced quiescence. *J. Exp. Biol.* **198**, 1299–1305.

Anchordoguy, T. J., Hofmann, G. E. and Hand, S. C. (1993). Extension of enzyme-half life during quiescence in *Artemia* embryos. *Am. J. Physiol.* **264**, R85–R89.

Atkinson, D. E. (1977). *Cellular Energy Metabolism and Its Regulation*. New York: Academic Press.

Bunn, H. F. and Poyton, R. O. (1996). Oxygen sensing and molecular adaptation to hypoxia. *Physiol. Rev.* **76**, 839–885.

Busa, W. B. and Crowe, J. H. (1983). Intracellular pH regulates transitions between dormancy and development of brine shrimp (*Artemia salina*) embryos. *Science* **221**, 366–368.

Busa, W. B., Crowe, J. H. and Matson, G. B. (1982). Intracellular pH and the metabolic status of dormant and developing *Artemia* embryos. *Arch. Biochem. Biophys.* **216**, 711–718.

Carpenter, J. F. and Hand, S. C. (1986). Arrestment of carbohydrate metabolism during anaerobic dormancy and aerobic acidosis in *Artemia* embryos: determination of pH-sensitive control points. *J. Comp. Physiol.* B **156**, 451–459.

Clegg, J. S. (1997). Embryos of *Artemia franciscana* survive four years of continuous anoxia: the case for complete metabolic rate depression. *J. Exp. Biol.* **200**, 467–475.

Clegg, J. S. and Golub, A. L. (1969). Protein synthesis in *Artemia salina* embryos. II. Resumption of RNA and protein synthesis upon cessation of dormancy in the encysted gastrula. *Dev. Biol.* **19**, 178–200.

Clegg, J. S. and Jackson, S. A. (1989). In *The Cellular and Molecular Biology of* Artemia *Development* (ed. A. H. Warner, J. Bagshaw and T. MacRae), pp. 1–15. New York: Plenum Press.

Clegg, J. S., Jackson, S. A., Liang, P. and MacRae, T. H. (1995). Nuclear–cytoplasmic translocation of protein p26 during aerobic–anoxic transitions in embryos of *Artemia franciscana*. *Exp. Cell Res.* **219**, 1–7.

Clegg, J. S., Jackson, S. A. and Warner, A. H. (1994). Extensive intracellular translocations of a major protein accompany anoxia in embryos of *Artemia franciscana*. *Exp. Cell Res.* **212**, 77–83.

Cornish-Bowden, A. (1995). *Analysis of Enzyme Kinetic Data*. Oxford: Oxford University Press.

D'Alessio, J. M. and Bagshaw, J. C. (1977). DNA-dependent RNA polymerases from *Artemia salina*. *Differentiation* **8**, 53–56.

Dang, C. V. and Semenza, G. L. (1999). Oncogenic alterations of metabolism. *Trends Biochem. Sci.* **24**, 68–72.

Egan, R. M., Brockman, J. A., Omer, K. W. and Woodward, J. G. (1994). Transcription of the murine class II Eb gene is regulated primarily at the level of transcriptional initiation. *Cell Immunol.* **156**, 537–543.

Furuichi, Y. (1981). Allosteric stimulatory effect of S-adenosylmethionine on the RNA polymerase in cytoplasmic polyhedrosis virus. *J. Biol. Chem.* **256**, 483–493.

Gaal, T., Bartlett, M. S., Ross, W., Turnbough, C. L. and Gourse, R. L. (1997). Transcription regulation by initiating NTP concentration: rRNA synthesis in bacteria. *Science* **278**, 2092–2097.

Goldberg, M. A., Dunning, S. P. and Bunn, H. F. (1988). Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein. *Science* **242**, 1412–1415.

Grieshaber, M. K., Hardewig, I., Kreutzer, U. and Pörtner, H.-O. (1994). Physiological and metabolic responses to hypoxia in invertebrates. *Rev. Physiol. Biochem. Pharmac.* **125**, 43–147.

Groudine, M., Peretz, M. and Weintraub, H. (1981). Transcriptional regulation of hemoglobin switching in chicken embryos. *Mol. Cell. Biol.* **1**, 281–288.

Grummt, I. and Grummt, F. (1976). Control of nucleolar RNA synthesis by the intracellular pools of ATP and GTP. *Cell* **7**, 447–453.

Guppy, M. and Withers, P. (1999). Metabolic depression in animals: physiological perspectives and biochemical generalizations. *Biol. Rev.* **74**, 1–40.

Hand, S. C. (1991). Metabolic dormancy in aquatic invertebrates. *Adv. Comp. Env. Physiol.* **8**, 1–50.

Hand, S. C. (1995). Heat flow is measurable from *Artemia franciscana* embryos under anoxia. *J. Exp. Zool.* **273**, 445–449.

Hand, S. C. (1998). Quiescence in *Artemia franciscana* embryos: reversible arrest of metabolism and gene expression at low oxygen levels. *J. Exp. Biol.* **201**, 1233–1242.

Hand, S. C. and Gnaiger, E. (1988). Anaerobic dormancy quantified in *Artemia* embryos: a calorimetric test of the control mechanism. *Science* **239**, 1425–1427.

Hand, S. C. and Hardewig, I. (1996). Downregulation of cellular metabolism during environmental stress: mechanisms and implications. *Annu. Rev. Physiol.* **58**, 539–563.

Hand, S. C. and Podrabsky, J. E. (1999). Bioenergetics of diapause and quiescence in aquatic animals. *Thermochim. Acta* (in press).

Hardewig, I., Anchordoguy, T. J., Crawford, D. L. and Hand, S. C. (1996). Profiles of nuclear and mitochondrial encoded mRNAs in developing and quiescent embryos of *Artemia franciscana*. *Mol. Cell. Biochem.* **158**, 139–147.

Hentschel, C. C. and Tata, J. R. (1977). Differential activation of free and template-engaged RNA polymerase I and II during resumption of development of dormant *Artemia* gastrulae. *Dev. Biol.* **57**, 293–304.

Hochachka, P. W., Buck, L. T., Doll, C. J. and Land, S. C. (1996). Unifying theory of hypoxia tolerance: Molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. *Proc. Natl. Acad. Sci. USA* **93**, 9493–9498.

Hochachka, P. W. and Guppy, M. (1987). *Metabolic Arrest and the Control of Biological Time.* Cambridge: Harvard University Press.

Hochachka, P. W., Lutz, P. L., Sick, T., Rosenthal, M. and van den Thillart, G. (1993). (eds) *Surviving Hypoxia. Mechanisms of Control and Adaptation*. Boca Raton, FL: CRC Press.

Hofer, E. and Darnell, J. E. (1981). The primary transcription unit of the mouse beta-major globin gene. *Cell* **23**, 585–593.

Hofmann, G. E. and Hand, S. C. (1990). Arrest of cytochrome-*c* oxidase synthesis coordinated with catabolic arrest in dormant *Artemia* embryos. *Am. J. Physiol.* **258**, R1184–R1191.

Hofmann, G. E. and Hand, S. C. (1992). Comparison of messenger RNA pools in active and dormant *Artemia franciscana* embryos: evidence for translational control. *J. Exp. Biol.* **164**, 103–116.

Hofmann, G. E. and Hand, S. C. (1994). Global arrest of translation during invertebrate quiescence. *Proc. Natl. Acad. Sci. USA* **91**, 8492–8496.

Hontoria, F., Crowe, J. H., Crowe, L. M. and Amat, F. (1993). Metabolic heat production by *Artemia* embryos under anoxic conditions. *J. Exp. Biol.* **178**, 149–159.

Job, D., Marmillot, P., Job, C. and Jovin, T. M. (1988). Transcription of left-handed Z-DNA templates: Increased rate of single-step addition reactions catalyzed by wheat germ RNA polymerase II. *Biochemistry* **27**, 6371–6378.

Klumpp, K., Ford, M. J. and Ruigrok, R. W. H. (1998). Variation in ATP requirement during influenza virus transcription. *J. Gen. Virol.* **79**, 1033–1045.

Kwast, K. E. and Hand, S. C. (1993). Regulatory features of protein synthesis in isolated mitochondria from *Artemia* embryos. *Am. J. Physiol*. **265**, R1238–R1246.

Kwast, K. E. and Hand, S. C. (1996a). Acute depression of mitochondrial protein synthesis during anoxia. *J. Biol. Chem.* **271**, 7313–7319.

Kwast, K. E. and Hand, S. C. (1996b). Oxygen and pH regulation of protein synthesis in mitochondria from *Artemia franciscana. Biochem. J.* **313**, 207–213.

Kwast, K. E., Shapiro, J. I., Rees, B. B. and Hand, S. C. (1995). Oxidative phosphorylation and the realkalinization of intracellular pH during recovery from anoxia in *Artemia franciscana* embryos. *Biochim. Biophys. Acta* **1232**, 5–12.

Land, S. C. and Hochachka, P. W. (1995). A heme-protein-based oxygen-sensing mechanism controls the expression and suppression of multiple proteins in anoxia-tolerant turtle hepatocytes. *Proc. Natl. Acad. Sci. USA* **92**, 7505–7509.

Lassam, N. and Jay, G. (1989). Suppression of MHC Class I RNA in highly oncogenic cells occurs at the level of transcription initiation. *J. Immunol.* **143**, 3792–3797.

Liang, P., Amons, R., Clegg, J. S. and MacRae, T. H. (1997a). Molecular characterization of a small heat shock/α-crystallin protein in encysted *Artemia* embryos. *J. Biol. Chem.* **272**, 19051–19058.

Liang, P. R., Amons, R. and Clegg, J. S. (1997b). Purification, structure and *in vitro* molecular-chaperone activity of *Artemia* p26, a small heat-shock/alpha-crystallin protein. *Eur. J. Biochem.* **243**, 225–232.

Liang, P. and MacRae, T. H. (1999). The synthesis of a small heat shock/alpha-crystallin protein in *Artemia* and its relationship to stress tolerance during development. *Dev. Biol*. **207**, 445–456.

Lohr, D. and Ide, G. I. (1983). *In vitro* initiation and termination of ribosomal RNA transcription in isolated yeast nuclei. *J. Biol. Chem.* **258**, 4668–4671.

Ratcliffe, P. J., O'Rourke, J. F., Maxwell, P. H. and Pugh, C. W. (1998). Oxygen sensing, hypoxia-inducible factor-1 and the regulation of mammalian gene expression. *J. Exp. Biol.* **201**, 1153–1162.

Rees, B. B., Ropson, I. J. and Hand, S. C. (1989). Kinetic properties of hexokinase under near-physiological conditions – relation to metabolic arrest in *Artemia* embryos during anoxia. *J. Biol. Chem.* **264**, 15410–15417.

Rolfe, D. F. S. and Brown, G. C. (1997). Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physiol. Rev.* **77**, 731–758.

Semenza, G. L., Roth, P. H., Fang, H. and Wang, G. L. (1994). Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J. Biol. Chem.* **269**, 23757–23763.

Shilatifard, A. (1998). Factors regulating the transcriptional elongation activity of RNA polymerase II. *FASEB J.* **12**, 1437–1446.

Somero, G. N. (1998). (ed.) The biology of oxygen: evolutionary, physiological and molecular aspects. *J. Exp. Biol.* **201**, 1043–1254.

Spencer, C. A. and Kilvert, M. A. (1993). Transcription elongation in the human c-myc gene is governed by overall transcription initiation levels in *Xenopus* oocytes. *Mol. Cell. Biol.* **13**, 1296–1305.

Stallcup, M. R., Ring, J. and Yamamoto, K. R. (1978). Synthesis of mouse mammary tumor virus ribonucleic acid in isolated nuclei from cultured mammary tumor cells. *Biochemistry* **17**, 1515–1521.

Stocco, D. M., Beers, P. C. and Warner, A. H. (1972). Effect of anoxia on nucleotide metabolism in encysted embryos of the brine shrimp. *Dev. Biol.* **27**, 479–493.

Storey, K. B. and Storey, J. M. (1990). Metabolic rate depression and biochemical adaptation in anaerobiosis, hibernation and estivation. *Q. Rev. Biol.* **65**, 145–174.

Uptain, S. M., Kane, C. M. and Chamberlin, M. J. (1997). Basic mechanisms of transcript elongation and its regulation. *Annu. Rev. Biochem.* **66**, 117–172.

Weber, J., Jelinek, W. and Darnell, J. E. (1977). The definition of a large viral transcription unit late in Ad2 infection of HeLa cells: Mapping of nascent RNA molecules labeled in isolated nuclei. *Cell* **10**, 611–616.